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April 6, 1999

Commissioner of Patents and Trademarks
U S Patent and Trademark Office
Washington, DC 20231

Dear Sir·

RE. New US Patent Application Entitled: BRCA1 AND hMLHI GENE PRIMER SEQUENCES AND METHOD FOR TESTING
Inventor Jan Vijg, of San Antonio, Texas
Corresponding to Provisional Application U.S.Serial No. 60/084408, May 6, 1998

We enclose the above-identified patent application, together with 2 (two) sheets of drawings, and a Verified Statement (small entity status). Declaration (Power of Attorney) will follow shortly

The Commissioner is hereby authorized to charge the filing fee and any additional fees that may be due, to the deposit account No. 18-1425 of the undersigned attorney,

Cordially,

RINES AND RINES

Robert H Rines

Robert H Rines

RHR/ipo--Enclosures

FORM 1

Applicant or Patentee: Jan Vijg
Serial or Patent No.: Corresponding to Provisional US Application
Filed or Issued: No. 60/084408, May 6, 1998
For: BRCA1 and hMLHI Gene Primer Sequences And Method For Testing

Attorney's Docket No.: _____

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS (37 CFR 1.9(f) and 1.27(b)) - INDEPENDENT INVENTOR

As a below named inventor, I hereby declare that I qualify as an independent inventor as defined in 37 CFR 1.9(c) for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code, to the Patent and Trademark Office with regard to the invention entitled as above described in

[ ] the specification filed herewith 60/084408
[x] application serial no. (Provisional)/_____, filed May 6, 1998
[ ] patent no. _____, issued _____.

I have not assigned, granted, conveyed or licensed and am under no obligation under contract or law to assign, grant, convey or license, any rights in the invention to any person who could not be classified as an independent inventor under 37 CFR 1.9(c) if that person had made the invention, or to any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

Each person, concern or organization to which I have assigned, granted, conveyed, or licensed or am under an obligation under contract or law to assign, grant, convey, or license any rights in the invention is listed below:

[ ] no such person, concern, or organization
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*NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

FULL NAME _____
ADDRESS _____
[ ] INDIVIDUAL [ ] SMALL BUSINESS CONCERN [ ] NONPROFIT ORGANIZATION

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I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Jan Vijg		
NAME OF INVENTOR	NAME OF INVENTOR	NAME OF INVENTOR
Robert H. Rines, Esq. Robert H Rines		
Signature of ~~Inventor~~ Counsel	Signature of Inventor	Signature of Inventor
May 6, 1999		
Date	Date	Date

BRCAI AND hMLHI GENE PRIMER SEQUENCES AND METHOD FOR TESTING

The present application is based upon provisional application 60/084408, filed May 6, 1998, and is directed to methods of and primer sequences for sequence variation and/or mutation detection of BRCA and hMLH1 genes , such as by two-dimensional denaturing gradient electrophoresis techniques (TDGS).

Background

Such techniques are described in Method Of And Apparatus For Diagnostic DNA Testing, Jan Vijg and Daizong Li, PCT/IB96/00543, filed 3 June 1996, International Publication Number WO96/39535, 12 December 1996, and in "Two-Dimensional DNA Typing", Molecular Bio Technolgoy, Vol. 4, 1995, pp 275-295

The tests leading to the establishment of the primer sequences for the BRCAI and hMLH1 of the present invention were conducted with the TDGS design prepared with the computer programming and equipment described in PCT/IB97/00976, published on or about February 14, 1998.

Objects of Invention and Summary

The objects of the invention are to provide novel theoretically and empirically (experimentally) derived TDGS patterns for hMLH1 and BRCA1 genes which may be used by testers to test for gene sequence variation and/or mutations.

Drawings

Figs. 1A and 1B show the computer-aided design TDGS patterns obtained for the hMLH1 and BRCA1 (theoretical-left hand side, empirical or experimental--right hand side)

In the theoretical vs. empirical patterns of the MLHI and BRCA1 genes, for all four genes, one or more exons were designed in overlapping fragments, in which case the fragment name is exon.1, exon..2, etc. Exons 8 and 15 of hMLH1 contain polymorphisms, which can be distinguished from disease-causing heterozygous mutations on the basis of a unique four-spot pattern (18).

Description Of The Invention In Preferred Forms

The MLH1 DNA mismatch repair gene. The design for *MLH1* took 30 minutes (excluding exon indication). Fig. 1A shows the theoretical and the empirical TDGS pattern for the *MLH1* gene. Because exons 11 and 12 had to be subdivided into overlapping fragments, two multiplex groups are currently being used, with the long PCR carried out as a four-plex PCR. Like many other genes, exon 1 of *MLH1* is GC-rich and, hence, was found to melt at a much higher % UF compared to most of the other fragments. Thus far, a total of 41 coded samples with previously identified mutations have been analyzed in a blinded fashion with 100% concordance (30).

The breast and ovarian cancer susceptibility gene BRCA1. The tumor suppressor gene *BRCA1* contains 24 exons, of which exon 11 contains approximately 60 % of the coding region. Fig. 1B shows the theoretical and empirical 2-D pattern for *BRCA1*. Of all 2-D designs discussed, this was the most difficult (total design time was 2 h), the main reason being the need to make overlapping fragments for the 3.4 kb exon 11. Pre-amplification was accomplished by one 7-plex long PCR. Using the long PCR amplicons as template, all 24 exons were amplified in a total of 37 fragments distributed over 5 multiplex groups. The overlap and sometimes short distances from fragment to fragment necessitated the use of so many multiplex short PCR groups. The non-coding exons 1a, 1b and the non-coding part of exon 24 were excluded. Evaluation of this test design using a panel of coded samples with previously identified mutations is currently ongoing. Thus far, mutations and polymorphisms have been detected in exons 2, 8, 11, 16, 20 and 23.

PCR Amplification

Primers were obtained from Genosys Biotechnologies, Inc. (The Woodlands, TX). For complete lists of all sequences, except *BRCA1*, see references 18, 29 and 30. Primer sequences for *BRCA1* will be published elsewhere but will be made available upon request. PCR amplification of gene sequences was carried out using the two-step protocol first described by Li and Vijg (22). Primers for long-distance PCR were designed based on published sequences (24-27) using Primer Designer 3, to amplify the entire gene-coding region for each of the 4 genes as a 1-plex (*TP53*), a 6-plex PCR (*RB1*), a 4-plex PCR (*MLH1*) or a 7-plex PCR (*BRCA1*). The LA PCR kit (Takara) was used for long PCR in a PTC-100 thermocycler (MJ Research). Multiplex short PCR was carried out using the long PCR products as template. Between 0.1 and 1.125 µM of each primer was used in a 50 µl reaction with 1 µl of long PCR product in 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 250 µM of each dNTP and 5 % formamide. Two and a half units of Taq DNA polymerase (Life Technologies) were added after an initial denaturation at 94 °C for 60 s. Cycling conditions for multiplex short PCR and concentrations of $MgCl_2$ varied among different genes and amplifications were carried out in a PTC-100 thermocycler (MJ Research).

Two-dimensional DNA electrophoresis

For *RB1*, 5 µl of multiplex short PCR was used per electrophoresis run. For *TP53*, *MLH1* and *BRCA1*, 5 µl of each of the different multiplex groups were combined. One tenth of a

volume of loading buffer (0.25 % xylene cyanol, 0.25 % bromophenol blue, 15 % ficoll and 100 mM Na_2EDTA) was added and the mixtures were loaded onto a 6.5 % (*TP53*) or 10 % (*RB1*, *MLH1* and *BRCA1*) PAA non-denaturing size gel (acrylamide: bisacrylamide = 37.5:1) in 0.5 x TAE buffer. The samples were electrophoresed for 5.3 h at 150 V (*RB1*), 5 h at 120 V (*TP53*) or 7.5 h at 140 V (*MLH1* and *BRCA1*) at 50 °C. After staining the gel with a mixture of equal amounts of SYBR-green I and II (Molecular Probes, Eugene, Oregon) for 20 min, the region containing all fragments of interest (usually between 100 and 600 bp) was cut out and loaded onto a denaturing gradient gel (DGGE). Gradients used were 0 to 50 % UF for *RB1*, 20 to 70 % UF for *TP53*, 25 to 70 % UF for MLH1 and 20 to 65% UF for BRCA1. The second orthogonal dimension was run for 12 h at 100V (RBI), 14 h at 120 V (TP53) or 16 h at 100 V (MLH1 and BRCA1). Spot patterns were visualized by SYBR-green staining using a FluorImager (Molecular Dynamics, Sunnyvale, California).

What is claimed is:

1. A method of enabling BRCA1 and h MLHI gene testing for gene sequence variation and/or mutations, that comprises, preparing appropriate test kit primers and solvents suitable for amplifying by long distance PCR the entire gene-coding region of each of BRCA1 and MLHI genes, to be followed by multiplex short PCR using the long PCR products as templates; providing in such test kit appropriate buffer and gel and solvent materials for use in electrophoresis in orthogonal dimensions for producing spot patterns indicative of gene sequence variations and/or mutations.

2. The method of claim 1 wherein the test kit is provided with non-denaturing gel and buffer materials suitable to enable combined mixtures of the multiplex groups of BRCA1 and MLHI to be subjected to the electrophoresis simultaneously together.

3. The method of claims wherein, the kits provide the Primer Pairs A and B listed in the specification for Long Distance and Short PCR, respectively.

4. Test kits for enabling BRCA1 and h MLHI gene testing prepared by the method of claim 3.

ABSTRACT

Primer sequences and materials are pre-prepared as test kits for enabling appropriate gene scanning patterns, preferably by two dimensional electrophoresis (TDGS), for use in detecting sequence variations and/or mutations in BRCA1 and h MLH1 genes

6 The primer sequences for long and short PCR for the BRCA1 are as follows:

A. Primer Pairs for Long Distance PCR

Exons 1-4

MLH1-4F GCG.GCT.AAG.CTA.CAG.CTG.AAG.GAA.GAA.CGT.GA

MLH1-4R GGC.GAG.ACA.GGA.TTA.CTC.TGA.GAC.CTA.GGC.CC

product size= 10.8kb

Exons 5-10

MLH5-10F

GCG.CCC.CTT.GGG.ATT.AGT.ATC.TAT.CTC.TCT.ACT.GG

MLH5-10R GCG.CTC.ATC.TCT.TTC.AAA.GAG.GAG.AGC.CTG

product size=10.5kb

Exons 11-13

MLH11-13F CGG.CTT.TTT.CTC.CCC.CTC.CCA.CTA.TCT.AAG.G

MLH11-13R GGG.TTA.GTA.AAG.GAA.GAG.GAG.CTT.GCC.C

product size=8.7kb

Exons 14-19

MLH14-19F GGT.GCT.TTG.GTC.AAT.GAA.GTG.GGG.TTG.GTA.G

MLH14-19R

GCG.CGC.GTA.TGT.TGG.TAC.ACT.TTG.TAT.ATC.ACA.C

product size=10.5kb

Underlined nucleotides represent nucleotides added to modify melting temperatures of the primers

B. Primer Pairs for Short PCR

Exon	Clamp[1]	Product Size	Tm[2]	Primer Sequence

12.1	40	184	44.53	TTT.TTT.TTT.TTT.TAA.TAC.A
				AAT.CTG.TAC.GAA.CCA.TCT
12.2	8	366	53.23	TGG.AAG.TAG.TGA.TAA.GGT
	40			TGT.ACT.TTT.CCC.AAA.AGG
13	40	272	49.06	ATC.TGC.ACT.TCC.TTT.TCT
				AAA.ACC.TTG.GCA.GTT.GAG
14	45	235	48.94	TAC.TTA.CCT.GTT.TTT.TGG
	5			GTA.GTA.GCT.CTG.CTT.GTT
15	40	179	29.97	CAG.CTT.TTC.CTT.AAA.GTC
				CAG.TTG.AAA.TGT.CAG.AAG
16		261	47.56	CTT.GCT.CCT.TCA.TGT.TCT.TG
	40			AGA.AGT.ATA.AGA.ATG.GCT.GTC
17	40	199	47.01	ATT.ATT.TCT.TGT.TCC.CTT
				AAT.GCT.TAG.TAT.CTG.CCT
18	45	215	46.67	CCT.ATT.TTG.AGG.TAT.TGA.AT
				GCC.AGT.GTG.CAT.CAC.CA
19.1		282	43.43	TGT.TGG.GAT.GCA.AAC.AGG
	40			ATC.CCA.CAG.TGC.ATA.AAT

[1]GC clamps:

50 clamp:

CGC.CCG.CCG.CCG.CCC.GCC.GCG.CCC.CGC.GCC.CGT.CCC.GCC.GC

C.CCC.GCC.CG

45 GC clamp:

CGC.CCG.CCG.CGC.CCC.GCG.CCC.GTC.CCG.CCG.CCC.CCG.CCC.GG
C.CCG

40 clamp:

CGC.CCG.CCG.CGC.CCC.GCG.CCC.GGC.CCG.CCG.CCC.CCG.CCC.G

8 clamp:

CGT.CCC.GC

5 clamp:

GCG.CG

2 clamp:

CG

[2]Tm is given in %UF

The primer sequences for long and short PCR for the BRCA1 are as follows:

Primers for long-PCR BRCA1
(7-PLEX)

BR1/1-3F : TgT ACC TTg ATT TCg TAT TCT gAg Agg CTg CTg CTT Ag
BR1/1-3R : gAg AAA gAA TgA AAT ggA gTT ggA TTT TCg TTC TCA C

Size: ***9.9 kb***

BR1/5-9F : TAg CCA TgA AAA gAT AAT CTC ACA ACT gCC CTT AAg AgC
BR1/5-9R : ACC AgC CTA CTT gAg ggA ggA Agg Tgg gAA gA

Size: ***9.7 kb***

BR1/10-11F : gAg AgC AgC TTT CAC TAA CTA AAT AAg ATT ggT CAg CTT TCT gT
BR1/10-11R : TCA AgT TTA AgA AgC AgT TCC TTT AAC TAT ACT Tgg AAA TTT gT

Size: ***4.8 kb***

BR1/12-13F : gCT Agg ACg TCA TCT TTg gCT TgA ATg AgC TTT A
BR1/12-13R : gCg ATA ATT ACC CAT gTg CTg AgC AAg gAT CA

Size: ***9.0 kb***

BR1/14-17F : TCT TCA ATg Tgg Agg CAg TAg ggA Tgg AgA AA
BR1/14-17R : ggg TCT CCA ggT TTT gCC TCA CTT gTT CTT TC

Size: ***10.7 kb***

BR1/18-20F : TCT TAA CTT CAT ATC AgC CTC CCC TAg ACT TCC AAA TAT CC
BR1/18-20R : CAT CTC TgC AAA ggg gAg Tgg AAT ACA gAg Tg

Size: ***7.2 kb***

BR1/21-24F : CAC TCT TCC ATC CCA ACC ACA TAA ATA AgT ATT gTC TC
BR1/21-24R : gCA TAg CCA gAA gTC CTT TTC Agg CTg ATg TAC AT

Size: ***11.4 kb***

10 Exon 11

BC1EX11

Exon	Frag	Primers 5' -> 3'		size	Tm(%UF)
11.1	[GC 3]	ACCTTGTTATTTTTGTATATTT	22	347	40.99
	[GC 13]	TTGCTAAGCCAGGCTGTT	18		
11.2	[GC 3]	ATACTCATGCCAGCTCATTA	20	461	40.74
	[GC 12]	AACGTCCAATACATCAGCTA	20		
11.3		CATGCTCAGAGAATCCTAGA	20	438	35.04
	[GC 3]	CTGTGGCTCAGTAACAAATG	20		
11.4	[GC 12]	TCACTCCAAATCAGTAGAGA	20	476	34.85
	[GC 3]	TACTGCTGCTTATAGGTTCA	20		
11.5	[GC 3]	GAAAGCAGATTTGGCAGTTC	20	468	33.66
	[GC 11]	CTGACTGGCATTTGGTTGTA	20		
11.6	[GC 3]	GAATAGGCTGAGGAGGAAGT	20	410	40.51
	[GC 13]	CTCTTGGAAGGCTAGGATTG	20		
11.7	[GC 3]	ACAGCGATACTTTCCCAGAG	20	345	36.45
		TGCCTTCCCTAGAGTGCTAA	20		
11.8		TTGCAAACTGAAAGATCTGT	20	365	38.37
	[GC 3]	GCTTTGAAACCTTGAATGTA	20		
11.9	[GC 13]	GTCGGGAAACAAGCATAGAA	20	422	40.40
	[GC 4]	TTGCCTCTGAACTGAGATGA	20		
11.10	[GC 12]	TAATATCACTGCAGGCTTTC	20	292	35.93
	[GC 1]	TTCCTCAAAGTTTTCCTCTA	20		
11.11	[GC 1]	TCCCATCAAGTCATTTGTTA	20	390	33.06
		TTCCAGGAAGACTTTGTTTA	20		
11.12	[GC 12]	TAATGAAGTGGGCTCCAGTA	20	309	33.22
	[GC 1]	CTTCCCATAGGCTGTTCTAA	20		
11.13	[GC 1]	GCAAGAATATGAAGAAGTAG	20	305	37.43
		CAAATGTGTATGGGTGAAAG	20		
11.14	[GC 1]	AGACACCTGATGACCTGTTA	20	378	43.03
	[GC 12]	TCTCCTCTGTGTTCTTAGAC	20		
11.15		CCTTTCACCCATACACATTT	20	460	39.33
	[GC 8]	GACTGATGCCTCATTTGTTT	20		
11.16	[GC 3]	CTCAGGAACATCACCTTAGT	20	356	44.00
	[GC 16]	ATAAATAGACTGGGCCACAC	20		

All exons excluding exon 11

BRCAONE

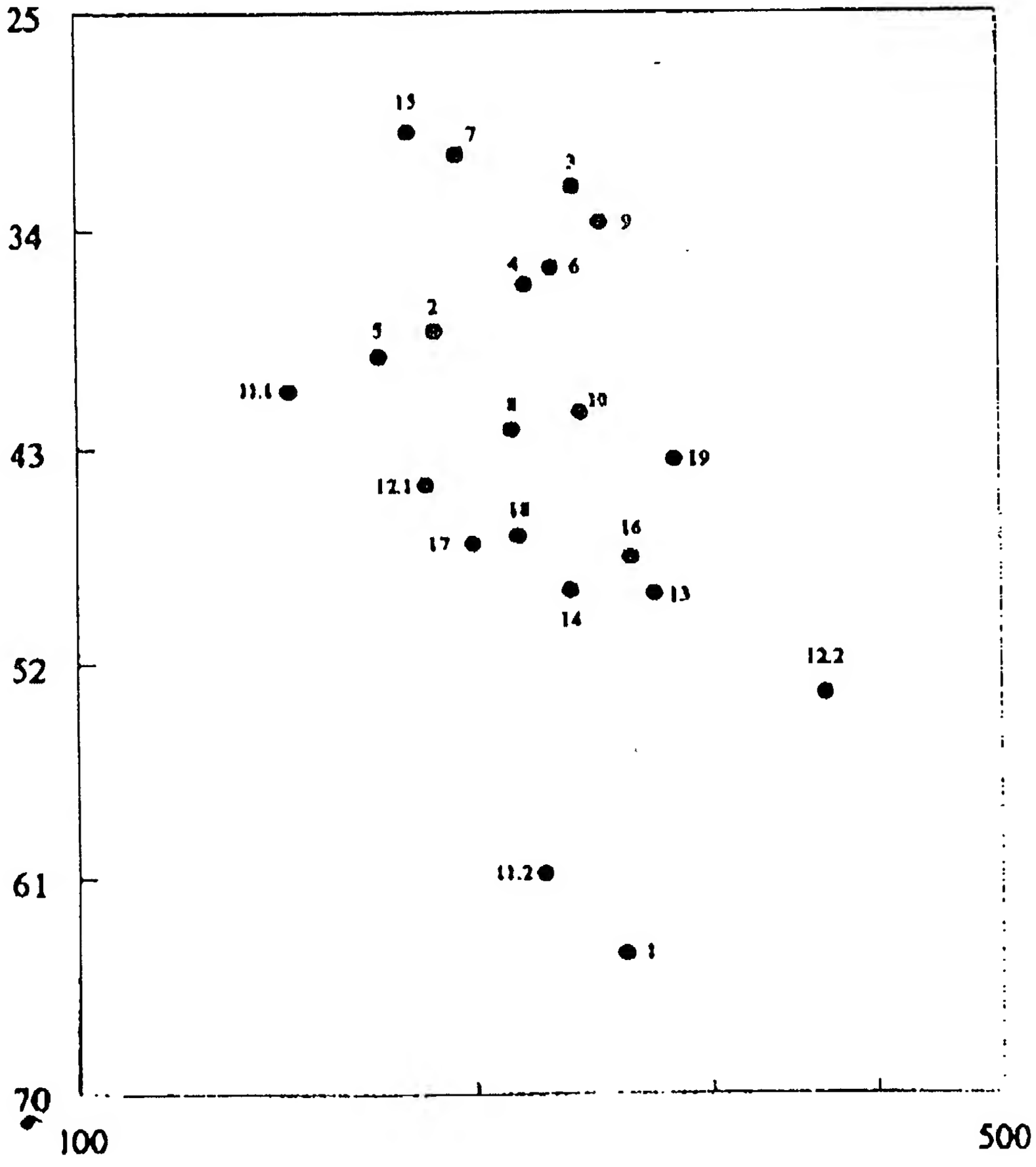
Exon	Frag	Primers 5' -> 3'		size	Tm(%UF)
2	1	[GC 1]TATATATGTTTTTCTAATGTGT	22	203	34.64
		[GC 12]TCCCAAATTAATACACTCTT	20		
3	1	[GC 12]GAGCCTCATTTATTTTCT	18	269	37.22
		[GC 4]ATTTTTCGTTCTCACTTA	18		
5	1	[GC 4]TATTTGCCTTTTGAGTAT	18	305	26.69
		[GC 12]TCTGATGAATGGTTTTAT	18		
6	1	[GC 8]ACTTGCTGAGTGTGTTTC	18	213	35.52
		GCACTTGAGTTGCATTCT	18		
7	1	[GC 3]TACATTTTTCTCTAACTGC	19	250	32.67
		GAAGAAAACAAATGGTTTT	19		
8	1	GGAGGAAAAGCACAGAAC	18	248	40.51
		[GC 3]CCAGCAATTATTATTAAATACTT	23		
9	1	[GC 3]CAGTAGATGCTCAGTAAA	18	242	24.26
		AATACCAGCTTCATAGAC	18		
10	1	[GC 4]CTGCATACATGTAACTAG	18	229	38.30
		CTACCCACTCTCTTTTCA	18		
12	1	[GC 4]AGTTGCAGCGTTTATAGT	18	289	48.54
		[GC 13]CAGCAAACCTAAGAATGT	18		
13	1	[GC 4]GCTTCTCAAAGTATTTCA	18	293	45.18
		AGTGTTTGGCCAACAATA	18		
14	1	[GC 4]CCAATTTGTGTATCATAG	18	417	30.78
		[GC 13]AGTGTATAAATGCCTGTA	18		
15	1	[GC 1]TGGTTTTCTCCTTCCATTTA	20	303	46.07
		[GC 16]TGTTCCAATACAGCAGATGA	20		
16	1	[GC 13]CGTTGTGTAAATTAAACTTC	20	427	47.49
		[GC 1]AGTCATTAGGGAGATACATA	20		
17	1	[GC 4]TGTGCTAGAGGTAACTCA	18	242	32.51
		[GC 11]CTCATGTGGTTTTATGCA	18		
18	1	[GC 12]TTTCAACTTCTAATCCTTT	19	194	36.32
		[GC 4]GGAGAAATAGTATTATACT	19		
19	1	GTTCTTCTGCTGTATGTA	18	178	32.32
		[GC 4]CTGAATGAATATCTCTGG	18		
20	1	[GC 4]CTCTTTCTCTTATCCTGAT	19	219	46.40
		TGGTGGGGTGAGATTTTT	18		
21	1	[GC 8]ATTCCCCTGTCCCTCTCT	18	172	49.95

CTGGAACTCTGGGGTTCT 18

2	1	[GC 4]TGATTTTACATCTAAATGTC 20 [GC 13]AGGAGAGAATATTGTGTC 18		209	47.71
3	1	[GC 12]TAGTCCTACTTTGACACT 18 [GC 4]AAATATTTAAAATGTGCCAA 20		275	49.47
4	1	[GC 13]AATCTCTGCTTGTGTTCTCT 20 [GC 18]ATTTAGTAGCCAGGACAGTA 20		325	59.79

FIG 1A

hMLH1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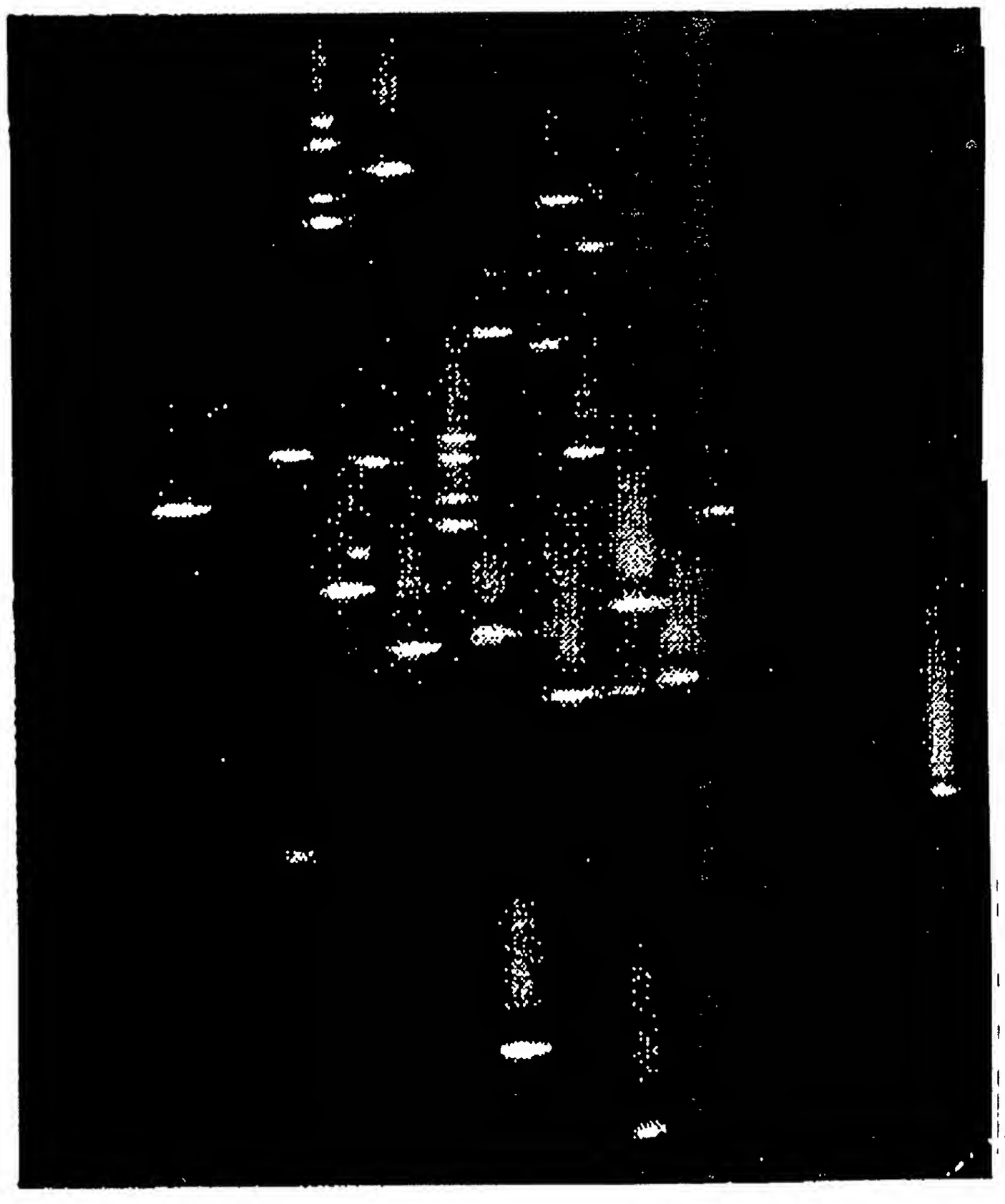

FIG. 1B

BRCA1